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REGULATION OF CREATINE PHOSPHOKINASE B ACTIVITY BY PROTEIN KINASE C

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Summary We previously reported that topical application of 12-O-tetradecanoylphorbol-13-acetate to mouse skin causes phosphorylation of epidermal proteins with molecular weights of 40,000 (p40) and 34,000 (p34). In the accompanying paper, p40 was identified as creatine phosphokinase B. Here we report that both in intact cells and in a cell-free system, phosphorylation of creatine hosphokinase B by protein kinase C resulted in an increase in its ability to catalyze the transfer of the high-energy phosphate of phosphocreatine to ADP, thereby producing ATP. H-7, a specific inhibitor of protein kinase C was found to abolish the increase in enzyme activity. Lineweaver-Burk plot analysis indicated that the increased activity was mostly due to a decreased Km for phosphocreatine. Phosphorylation and activation of creatine phosphokinase B may be a physiological response to maintain ATP balance when a protein kinase C pathway is stimulated.

Introduction Much attention has been paid to the transduction of extracellular signals into cells. One of the main pathways of signal transduction is that mediated by protein kinase C (PKC), as it is activated endogenously by diacylglycerol, a product of phosphatidylinositol turnover (1,2). Furthermore, PKC is a cellular receptor for phorbol ester tumor promoters, e.g. 12-O-tetradecanoylphorbol-13-acetate (TPA), which bind to and activate PKC. Activation of PKC then results in phosphorylation of target proteins, leading to expression of certain specific genes. Thus, identification of substrates for PKC is important to elucidate the biochemical mechanisms underlying the regulation of cellular functions and also of tumor promotion.

We previously demonstrated that cytosolic proteins of mouse epidermis with molecular weights of 40,000 and 34,000 (p40 and p34, respectively) were significantly phosphorylated at the serine residue when mouse skin in

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The abbreviations used are: PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; CKB, creatine phosphokinase B; PMSF, phenylmethylsulfonyl fluoride.

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vivo or BALB/MK-2 mouse epidermal keratinocyte were treated with TPA (3). In the accompanied paper, we purified p40 from mouse brain by the use of ^{32}P-labeled p40 of BALB/MK-2 cells as a tracer. Four lines of evidence indicate that p40 is creatine phosphokinase B (CKB). 1) The amino acid sequences of all peptide fragments of p40 from mouse brain were located in the primary structure of CKB. 2) p40 of BALB/MK-2 cells was immunoprecipitated with antibody against human CKB. 3) p40 of BALB/MK-2 cells was absorbed to and eluted from a creatin-affinity column. 4) Purified CKB was phosphorylated in vitro by purified PKC.

In this paper, we report that phosphorylation of CKB by PKC increases its activity both in intact cells and in a cell-free system.

MATERIALS AND METHODS

Cells. BALB/MK-2 epidermal keratinocytes (4) were provided by Dr. S. A. Aaronson, NCI, MD. Culture conditions were described in the accompanied paper.

Dephosphorylation and Phosphorylation of CKB. CKB was purified from mouse brain by a modification of the method of Roberts (5). Purified CKB was dephosphorylated by alkaline phosphatase conjugated with agarose beads and then phosphorylated in vitro by purified PKC from mouse brain (6) by the procedure described in the accompanied paper.

Assay of Creatine Phosphokinase. Creatine phosphokinase was assayed by monitoring the formation of NADPH from ATP in the presence of hexokinase and glucose-6-phosphodehydrogenase by the method of Roberts (5). In an intact cell system, quiescent cultures of BALB/MK-2 cells in 60 mm-dishes were treated with 100 ng/ml of TPA for the indicated times. The cells were lysated with a solution containing 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM 2-mercaptoethanol and 1% Triton X-100 and assayed. In a cell free system, dephosphorylated CKB was incubated with PKC in the presence or absence of 0.1 mM of H-7 (Seikagaku Kogyo, Tokyo, Japan) as described above. After precipitating the complex of phosphatidylserine, TPA and PKC by centrifugation at 100,000 x g for 1 h, the supernatant containing CKB was applied to a PD-10 column to remove H-7 and ATP and then to a DE-52 column (50 µl bed volume) equilibrated with 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 0.5 mM PMSF and 5 mM 2-mercaptoethanol. The column was washed with 120 mM NaCl and CKB was eluted with 400 mM NaCl. After adjusting to 200 mM NaCl, the enzyme solution was passed through a column of threonine-conjugated Sepharose equilibrated with 200 mM NaCl in the above buffer, to remove free PKC. The resulting solution, which did not contain ATP, H-7 or PKC, was assayed for creatine phosphokinase.

RESULTS

Activity of CKB was measured by formation of ATP by the transfer of the high-energy phosphate of phosphocreatine to ADP. When quiescent BALB/MK-2 cultures were treated with 100 ng/ml of TPA, a significant increase ($P < 0.01$) in enzyme activity was noted (Fig. 1). The increase was rapid, appearing within 5 min, with maximal activity 15 min after the treatment, and returning to the normal level after 120 min. Under these conditions, phosphorylation of p40 was in parallel with the activity of

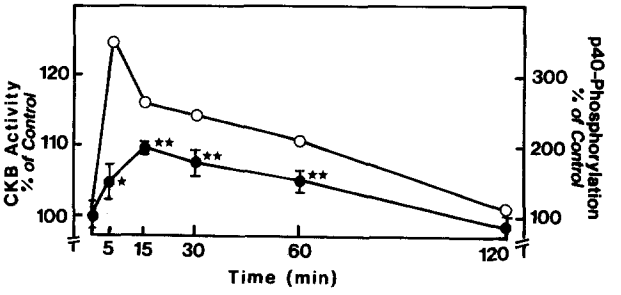

Fig. 1. Increase in CKB-activity (●) and in phosphorylation of p40 (○) in TPA-treated BALB/MK-2 cells. CKB activities are means ± SDs for 6 samples with percentages of that at 0 time (100% = 5.37 ±μmol NADPH formed). *, **, significant difference from value at 0 time at $P < 0.05$ and $P < 0.01$, respectively. p40-Phosphorylation is expressed as percentage of control (arbitrary unit).

CKB, reaching a peak 5 min after treatment and then gradually decreased (Fig. 1).

For assay in a cell-free system, purified CKB was dephosphorylated by alkaline phosphatase conjugated with agarose beads and then phosphorylated by PKC. As shown in Fig. 2, phosphorylation by PKC of the dephosphorylated CKB resulted in a 33% increase in its activity. This increase in activity on incubation with PKC was prevented by H-7, a specific inhibitor of PKC (7).

We found that activation of CKB by PKC was due to increase in affinity for phosphocreatine, a substrate of the enzyme (Fig. 3). Lineweaver-Burk plots with various concentrations of phosphocreatine indicated that the apparent Km value decreased from 2.6mM to 1.7mM on PKC-phosphorylation, with a slight increase in the Vmax value (7.7 and 9.3 μmol NADPH/min for the dephosphorylated and PKC-phosphorylated enzymes, respectively).

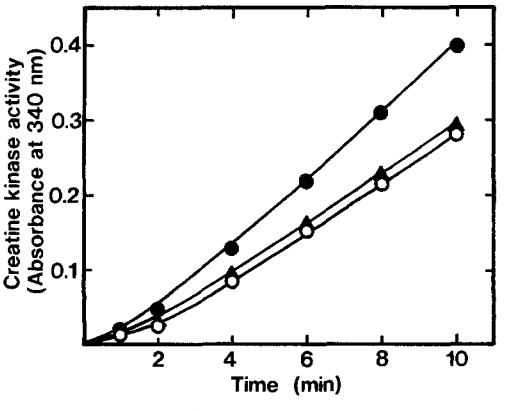

Fig. 2. Changes in CKB activities with time of incubation. ○, dephosphorylated CKB; ●, PKC-phosphorylated CKB; ▲, PKC-treated CKB in the presence of H-7.

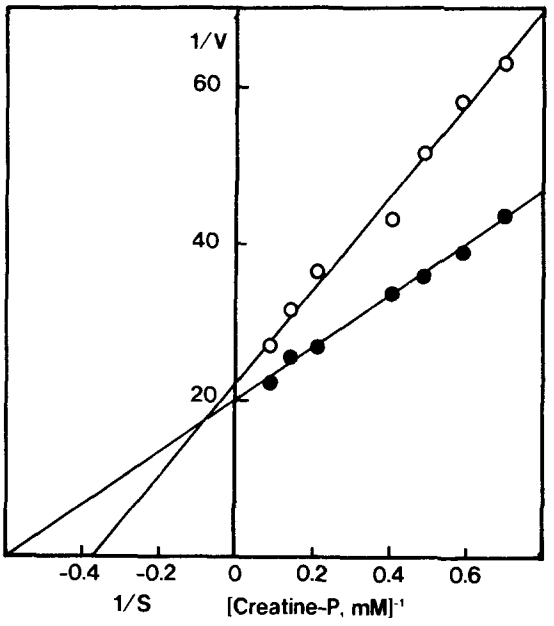

Fig. 3. Lineweaver-Burk plots of dephosphorylated (○) and PKC-phosphorylated CKB (●) vs concentration of phosphocreatine, a substrate of the enzyme.

DISCUSSION

In the present study, we found that phosphorylation of CKB by PKC increased its activity both in intact cells and in a cell-free system, and that this increase in activity was mostly due to an increase in its affinity for phosphocreatine, a substrate of the enzyme. Consistent with the present study, a recent report by Quest et al (8) demonstrated that phosphorylated chick CKB had a lower Km value for phosphocreatine, although a protein kinase responsible for this phosphorylation was not identified. A change in kinetics after phosphorylation was also demonstrated for inositol triphosphate 5'-phosphomonoesterase, the Vmax of which was increased by phosphorylation with PKC (9).

In the present paper, purified CKB was dephosphorylated prior to phosphorylation in vitro by PKC in order to eliminate possible endogenous phosphorylation. The previous observation that ^{32}Pi was incorporated, though weakly, into p40 in acetone-treated skin (3) suggests endogenous phosphorylation by protein kinases other than PKC. However, in the accompanied paper, we demonstrated that this endogenous phosphorylation seems not due to cAMP-dependent protein kinase or casein kinase II.

Regulation of CKB activity by PKC may be significant in maintaining the balance of ATP in cells. The pleiotropic effects of phorbol ester tumor promoters may reflect increased cellular activities such as increased cell

motility and growth, all of which require ATP as a source of energy. When there is a demand for ATP, PKC may act to prevent its rapid depletion by activating CKB. When cells are less active and the demand for ATP is not so high, this enzyme forms phosphocreatine as a reservoir of high-energy phosphate.

PKC is now known to consist of a family of related gene products and can be classified into two major types, PKC and nPKC, the latter of which is independent of Ca^{2+} for activation (1, 2). Little is known, however, about the tissue-specificity of PKC-isoforms and isoform-specific phosphorylation of substrates. Skin, or the keratinocytes of which the epidermis is composed, seems to be a unique tissue for studying these specificities in signalling pathways mediated by PKC.

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